S/N 09/503,559

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Roland Valdes, Jr. et al.	Examiner: Ulrike Winkler
Serial No.:	09/503,559	Group Art Unit: 1648
Filed:	February 11, 2000	Docket: 1160.003US1
Title:	DIHYDROOUABAIN-LIKE FACTOR AND DIAGNOSTIC & THERAPEUTIC COMPOSITIONS AND METHODS	

SUPPLEMENTAL DECLARATION OF DR. ROLAND VALDES, JR. UNDER 37 C.F.R. § 1.132

1. I, Roland Valdes, Jr., am one of the co-inventors of the above-identified patent application and am currently a Professor in the Department of Pathology and Laboratory Medicine at The University of Louisville in Louisville, Kentucky.

2. Physical properties related to molecular polarity and solubility of lactone hydrogenated ouabain-like factor (Dh-OLF) isolated from different mammalian sources can be distinguished by HPLC analysis. Our chromatographic reverse-phase HPLC method uses an isocratic mode of 10% CH_3CN in H_2O to separate the Dh-OLF and dihydrouoabain-B entities with baseline resolution. This technique also separates the respective deglycolylated genins (aglycones). We report two important findings: 1) Dh-OLF isolated from human serum does not separate from Dh-OLF isolated from bovine adrenal glands; and 2) Dh-OLF isolated from human serum chromatographically separates from the plant-derived dihydroouabain isomer B (dho-B). These data demonstrate that Dh-OLF extracted form human serum is similar to that extracted from bovine adrenal glands and, of importance, that Dihydro-OLF is different from the plant-related counterpart, dihydroouabain (dho-B). These data strongly indicate that mammalian species make a common Dh-OLF.

3. Chromatographic co-migration of human serum Dh-OLF and bovine adrenal Dh-OLF. In these experiments purified Dh-OLF from humans serum and from bovine adrenal cortex were mixed together and co-injected on HPLC using an isocratic 10%

CH_3CN mobile phase. The two molecules showed a single band of elution time of 26 minutes (**Figure 1, attached**). This same HPLC technique has previously been demonstrated to separate Dh-OLFs with fine structural resolution (see also below). Thus, co-migration using these techniques is consistent with material from these two distinct mammalian sources being similar compounds.

4. Chromatographic separation of mammalian Dh-OLF from plant-derived dho compounds. Similarly, pure human serum Dh-OLF and bovine adrenal cortex Dh-OLF (Qazzaz et al., Endocrinology, 2000;141(9):3200-3209) and their plant related counterpart Dihydroouabain-isomer B (pure Dho-B obtained from HPLC separation of dihydroouabain commercial preparation, Qazzaz et al., Biochem Biophys Acta, 1999:1472:486-497) were mixed together and co-injected on HPLC using an isocratic 10% CH_3CN mobile phase. The three molecules showed two bands separated by a minimum of 1 minute (**Figure 2, attached**). The first band (26 min) was identified (see above) as a co-eluting mixture of the two sources of Dh-OLF (human serum and adrenal cortex, see **Figure 1, attached**) and the second band eluted at 28 min representing dho-B. Similarly, the genin compounds (aglycone without the sugar molecules) of both parents (human serum Dh-OLF and Dho-B) when mixed and injected on HPLC also clearly separated by 1 to 1.5 minutes using the same isocratic HPLC mode. While the parent compounds, human serum Dh-OLF and Dho-B eluted at 26 and 28 respectively those of their genin components (human serum Dh-OLF-genin and dihydroouabain-B-genin) eluted at 18 and 20 minutes respectively (**Figure 3, attached**). This demonstrates beyond doubt that the mammalian-derived Dh-OLF is chromatographically distinct from the plant-derived dihydroouabain.

5. Methods. To remove the rahmonse moiety we treated the parent molecules [Dh-OLF (both sources) and Dho-B] individually (separately) with 1% SSA for 15 seconds and immediately separated the acid using a small C-18 reversed-phase Sep-Pak solid-phase extraction cartridge column previously wetted with acetonitrile (ACN) and rinsed with deionized water. These columns were eluted with 2 ml of 100% acetonitrile. To

remove the ACN, we evaporated the Sep-Pak eluents to dryness in a Savant Speed Vac, dissolved the residue in 1 ml deionized water, and filtered the solution through a Whatman 0.22-μm PVDF filter in preparation for HPLC.

6. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that the statements are made with the knowledge that willful false statement and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

11/28/00
Date

Roland Valdes, Jr.

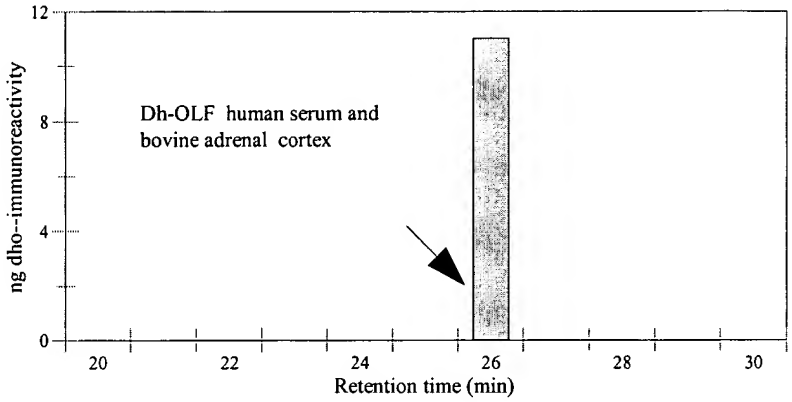

Figure 1. Chromatographic mobility of Dh-OLF isolated from human serum and bovine adrenal cortex mixed together

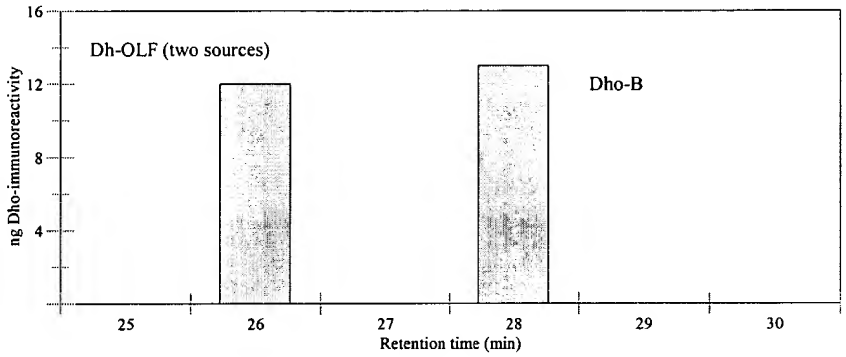

Figure 2. Chromatgraphic mobility of Dh-OLF (human serum) and Dh-OLF (adrenal Cortex) and plant related Dho-B

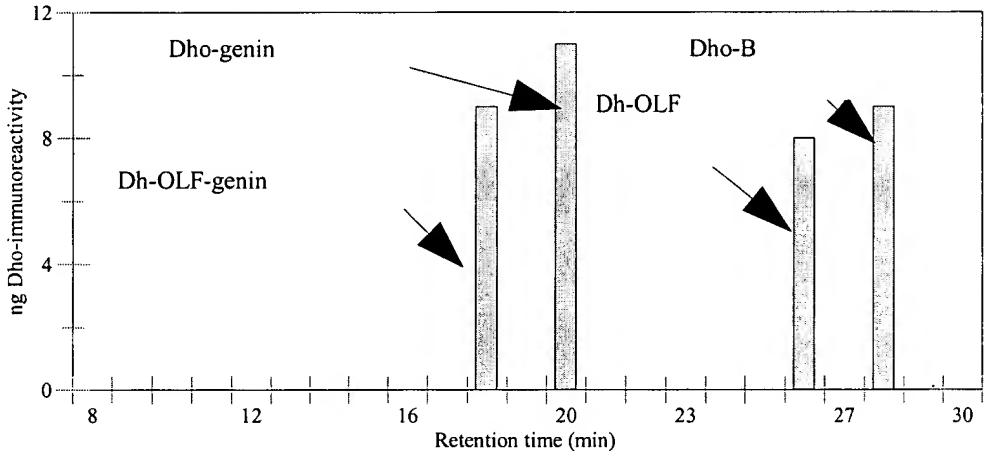

Figure 3. Chromatographic mobility of human serum Dh-OLF and dho-B and their congeners